REMARKS

Status of the Claims

Claims 1-3, 7, 8, 10-18, and 26-36 are pending in the present application. Claims 1(g) and 32 have been amended to recite that the nucleotide sequence encoding a *Lepidopteran* insect receptor polypeptide having *Bt* toxin binding activity is a nucleotide sequence encoding a polypeptide having the ligand binding sequence of SEQ ID NO:2. Claim 7 has been amended to recite a *Lepidopteran* insect receptor polypeptide consisting of at least 150 contiguous residues of the amino acid sequence set forth in SEQ ID NO:2, wherein said polypeptide comprises the ligand binding site of SEQ ID NO:2. Support for these amendments may be found on lines 5-10 of page 35 of the specification. No new matter has been added by amendment. Reexamination and reconsideration of the claims are respectfully requested.

The Examiner is respectfully requested to withdraw the rejections and allow claims 1-3, 7, 8, 10-18, and 26-36. In any event, the Examiner is requested to enter the above amendments for purposes of further prosecution. These amendments were not made earlier because Applicants earnestly believe that the specification is enabling for the breadth of the claims as originally drafted.

Consideration Of Previously Submitted Information Disclosure Statement

As previously mentioned in Applicants' response of January 16, 2003, the Examiner has not initialed citation number 25 on the PTO Form 1449 that was submitted with Applicants' Information Disclosure Statement mailed April 16, 2001. The Examiner returned the PTO form 1449 with the Office Action mailed October 18, 2002, but indicated that no copy of citation number 25, the Metty reference, was available to review. Applicants attached to the Amendment of January 16, 2003, a copy of the Information Disclosure Statement, the Form 1449, and the return postcard indicating that the OIPE received copies of both references cited on the Form 1449 on April 20, 2001. In addition, in order to facilitate review, a replacement copy of the Matty reference was also provided. It is requested that an initialed copy of the Form 1449 be forwarded to the undersigned with the next communication from the PTO.

RTA01/2135196v1

It is also noted that an initialed copy of the PTO Form 1449 that was submitted with Applicants' Information Disclosure Statement mailed January 31, 2002 has not been returned to Applicants' representative with the Office Action. Accordingly, it is requested that an initialed copy of the Form 1449 be forwarded to the undersigned with the next communication from the PTO. In order to facilitate review of the references by the Examiner, a copy of the Information Disclosure Statement, the Form 1449, and the return postcard indicating receipt by the OIPE on February 15, 2002 were attached to our Amendment of January 16, 2003. A copy of the cited references was provided at the time of filling the original Information Disclosure Statement, and, therefore, an addition copy of the references is not submitted herewith. Applicants will be pleased to provide an additional copy of the reference upon the Examiner's request if it proves difficult to locate the original reference.

The Rejection Under 35 U.S.C. § 112, First Paragraph, Should be Withdrawn

The Examiner has rejected claim 1 and dependent claims 2, 3, 7, 8, 10-18, and 32-36 on the grounds that they contain subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors had possession of the claimed invention at the time the application was filed. The rejection is respectfully traversed for the reasons described below.

The Examiner states that the claims reciting either a given percent identity or hybridization claim language and appropriate functional language do comply with the Written Description Guidelines. However, the Examiner argues that claims 1(g), 7(g), and 32 as previously amended do not meet the requirement for a written description because they recite nucleotide sequences encoding a *Lepidopteran* insect receptor polypeptide having *Bt* toxin binding activity, where the nucleotide sequence consists of at least 22 contiguous nucleotides of SEQ ID NO:1 or encodes at least 25 contiguous amino acids of SEQ ID NO:2, and therefore encompass sequences that do not contain a complete toxin binding domain.

As noted in Applicants' amendment mailed January 16, 2003, claims 1(g), 7(g), and 32 as previously amended encompass only those nucleotide sequences that have the recited structural

features and encode receptor polypeptides having *Bt* toxin binding activity. In addition, lines 5-10 of the specification provide guidance regarding the functional domains of the *Bt* toxin receptor of SEQ ID NO:2, showing the correlation between the structural and functional features of the receptor. Thus, the claims provide the relevant, identifying structural and functional characteristics of the members of each claimed genus of nucleotide sequences that distinguish them from other sequences. Accordingly, one skilled in the art would be able to determine the identity of the members of the claimed genera, and would recognize that the Applicants were in possession of the sequences recited in the claims.

Nevertheless, in order to expedite prosecution, Applicants have amended claims 1(g) and 32 to recite that the nucleotide sequence encoding a *Lepidopteran* insect receptor polypeptide having *Bt* toxin binding activity is a nucleotide sequence encoding a polypeptide having the ligand binding sequence of SEQ ID NO:2. Claim 7(g) has been amended to recite a *Lepidopteran* insect receptor polypeptide consisting of at least 150 contiguous residues of the amino acid sequence set forth in SEQ ID NO:2, wherein said polypeptide comprises the ligand binding site of SEQ ID NO:2. Support for these amendments may be found on lines 5-10 on page 35 of the specification.

Claim 1 and dependent claims 2, 3, 7, 8, 10-18, and 32 have been rejected under 35 U.S.C. § 112, first paragraph on the grounds that the specification does not enable one of skill in the art to make and use the claimed invention. The rejection is respectfully traversed for the reasons described below.

The Applicants do not agree that it would required undue experimentation for one of skill in the art to make and use the nucleotide sequences recited in claims 1(g), 7(g), and 32 as previously amended. The previously-amended claims encompass only those nucleotide sequences that encode receptor polypeptides having *Bt* toxin binding activity. Accordingly, non-functional receptors are not encompassed by the claims.

Furthermore, the references cited by the examiner, Skolnick and Fetrow (2000) *Trends in Biotechnology* 18:34-39 and Rudinger in *Peptide Hormones,* J.A. Parsons Ed. University Park

Press, Baltimore, June 1976, do not support the argument that undue experimentation would be required to make and use the invention of claims 1(g), 7(g), and 32 as previously amended. The Examiner has cited Skolnick and Fetrow, for the teaching that: (1) some proteins have multiple functional domains, making function prediction based on sequence more difficult; (2) proteins gain and lose function during evolution, and that (3) inaccurate use of sequence-to-function methods has lead to errors in functional annotations in sequence databases. However, the teachings of Skolnick and Fetrow are not relevant to the enablement of claims 1(g), 7(g), and 32 as previously amended because the claims are not directed to nucleotide sequences encoding proteins whose function is to be determined de novo from its sequence. Rather, the claims are directed to nucleotide sequences encoding fragments of a receptor having known activity, where the functional domains of the receptor are described in the specification.

Rudinger is not relevant to the enablement of claims 1(g), 7(g), and 32 as previously amended because it reflects the view of those of skill in the art in 1976, while the priority document for the present application was filed in 1999. As described by Applicants in the Amendment mailed January 16, 2003, Rudinger predates essentially the entire field of modern molecular biology. In response to Applicants' arguments showing the inapplicability of Rudinger, the Examiner argues that "no documentary evidence to the contrary has been made of record, nor has any evidence been provided that teaches away from that taught by Rudinger" (April 7, 2003 Office Action, page 4). Applicants note that MPEP § 2164.04 provides that the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. This is in accordance with *In re Marzochhi*, 169 USPQ 367 (CCPA 1971), where the court held that "it is incumbent on the Patent Office, whenever a rejection on this basis [enablement] is made to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertion of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 169 USPQ at 370.

However, in order to demonstrate the extensive changes in the state in the art that occurred subsequent to the publication of the Rudinger reference, Applicants cite Gayle *et al.*

(1993) *J. Biol. Chem.* 268:22105-22111, provided herewith as Appendix A for the convenience of the Examiner. This reference demonstrate the state of the art for making and testing variants of a polypeptide in 1993, six years before the filing of the priority document for the present application. Gayle *et al.* describe saturation mutagenesis of the mature human interluekin-1α (IL-1α) sequence. The authors report that more than 3,500 mutants of IL-1α were produced and analyzed to determine their biological activity and their binding activity, demonstrating that such mutants can be made and tested for function without undue experimentation. Furthermore, the authors state that "[m]ost of the molecule could be mutated with little effect on either [biological or binding] activity." Gayle *et al.*, page 22109, column 2. Thus, the Gayle *et al.* reference demonstrates that making functional variants of a polypeptide is routine to those of skill the art, and that many or most of the variants produced by amino acid substitution retain the biological activity of the native protein.

Accordingly, while some quantity of experimentation would be required to produce functional *Bt* toxin receptors meeting the structural limitations of claims 1(g), 7(g), and 32 as previously amended, the level of experimentation would not be undue in view of the nature of the invention, the state of the prior art (where *Bt* toxin receptor functional domains and activities have been described), the relative skill of those in the art (to whom the making and testing of variants is routine), the predictability in the art, the amount of direction provided in the specification (which provides guidance regarding preferred types of amino acid substitutions and describes assays for identifying functional *Bt* toxin receptors), the breadth of the claimed invention (for which the scope is defined by both structural and functional limitations), and the existence of several working examples of functional *Bt* toxin receptors. These factors support the conclusion that one of skill in the art could practice the claimed invention without undue experimentation.

Nevertheless, in order to expedite prosecution, Applicants have amended claims 1(g) and 32 to recite that the nucleotide sequence encoding a *Lepidopteran* insect receptor polypeptide having *Bt* toxin binding activity is a nucleotide sequence encoding a polypeptide having the

ligand binding sequence of SEQ ID NO:2. Claim 7(g) has been amended to recite a *Lepidopteran* insect receptor polypeptide consisting of at least 150 contiguous residues of the amino acid sequence set forth in SEQ ID NO:2, wherein said polypeptide comprises the ligand binding site of SEQ ID NO:2. Support for these amendments may be found on line 5 of page 16 and lines 5-10 of page 35 of the specification.

In view of the above amendments, all grounds for rejection under 35 U.S.C. § 112, first paragraph, have been overcome. Accordingly, reconsideration and withdrawal of the rejections are respectfully requested.

The Rejection Under 35 U.S.C. 112, Second Paragraph, Should be Withdrawn

Claims 7 and 8 have been rejected under 35 U.S.C. § 112, second paragraph, on the grounds that they are ambiguous for reciting "at least one polypeptide of interest." The Examiner further notes that Applicants did not address this rejection in the Amendment mailed January 13, 2003. Applicants acknowledge this error and sincerely regret any inconvenience caused by this omission. The rejection is respectfully traversed for the reasons described below.

The specification of the present application provides guidance regarding the recited polypeptides of interest on line 10 of page 21 through line 16 of page 22. Polypeptides of interest are described as including heterologous polypeptides having heterologous toxin binding domains, and a number of specific examples of such heterologous polypeptides are listed on lines 4-8 of page 22 of the specification. The specification also describes the use of cadherin superfamily members as the polypeptide of interest in the claimed fusion protein. Accordingly, one of skill in the art, when reading the claims in light of the supporting specification, would be able to ascertain with a reasonable degree of precision and particularity the area set out and circumscribed by the claims.

In view of the above arguments, all grounds for the rejection under 35 U.S.C. § 112, second paragraph, have been overcome. Reconsideration and withdrawal of the rejection are respectfully requested.

The Rejection Under 35 U.S.C. § 102(b) Should be Withdrawn

The rejection of claims 1-3, 7, 8, and 10-16 under 35 U.S.C. § 102(b) has been maintained on the grounds that the claimed invention is anticipated by U.S. Patent No. 5,693,491. The rejection is respectfully traversed as applied to the amended claims.

In order to expedite prosecution, claim 1 has been amended to delete the reference to hybridizing nucleotide sequences. Accordingly, U.S. Patent No. 5,693,491 does not anticipate claim 1 as amended.
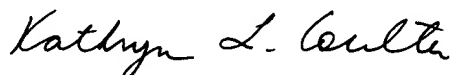
In view of the above amendments, all grounds for rejection under 35 U.S.C. § 102(b) have been overcome. Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

It is believed that all the rejections have been obviated or overcome and the claims are in conditions for allowance. Early notice to this effect is solicited. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject Application, the Examiner is invited to call the undersigned.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,
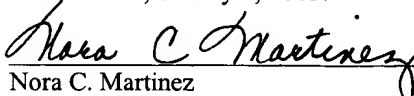
Kathryn L. Coulter

Kathryn L. Coulter
Registration No. 45,889

| **Customer No. 00826**
ALSTON & BIRD LLP
Bank of America Plaza
101 South Tryon Street, Suite 4000
Charlotte, NC 28280-4000
Tel Raleigh Office (919) 862-2200
Fax Raleigh Office (919) 862-2260 | CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to Mail Stop AF, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on July 7, 2003.

Nora C. Martinez
Nora C. Martinez |

In re: Flannagan et al.
Appl. No.   09/715,909
Filed   November 17, 2000

APPENDIX A

Identification of Regions in Interleukin-1α Important for Activity*

Richard B. Gayle, III‡§, Kurt Poindexter‡, David Cosman‡, Steven K. Dower‡, Steven Gillis‡, Thomas Hopp‡¶, Rita Jerzy‡, Shirley Kronheim‡, Vanessa Lum‖, Andrew Lewis‡, Marvin M. Goodgame‖, Carl J. March‡, Douglas L. Smith‖, and Subhashini Srinivasan‡

From the ‡Immunex Research and Development Corporation, Seattle, Washington 98101 and the ‖Life Sciences Research Laboratories, Eastman Kodak Company, Rochester, New York 14650

Saturation mutagenesis of the mature human interleukin-1α (IL-1α) gene has been performed. Following expression in *Escherichia coli*, the biological and receptor binding activities of the mutant proteins were examined. Most of the molecule could be altered with little effect on either function. More than 3,500 mutants were examined, and only 23 unique amino acid sequences were identified which resulted in an altered ratio of biological to binding activity when compared with wild-type IL-1α. These proteins possessed mutations at 38 of the 159 amino acid residues in IL-1α. Random mutagenesis at several of these positions identified further substitutions that affected activity. Examination of a model for IL-1α localized most of the residues which altered activity along one face of the molecule. This region appears to be distinct from areas of IL-1 which have been postulated to make contact with IL-1 receptor.

Interleukin-1 (IL-1)[1] is a potent cytokine that is involved in inflammatory responses and affects the growth and differentiation of T cells, B cells, and fibroblasts (for review, see Durum *et al.*, 1985). The two molecules responsible for this activity, IL-1α and IL-1β, share only 22% amino acid similarity (March *et al.*, 1985; Auron *et al.*, 1987). Each binds to both forms of IL-1 receptor (Dower *et al.*, 1986; McMahan *et al.*, 1991). Both IL-1 molecules are produced as intracellular precursors and are subsequently processed to mature proteins. Although the precursor form of IL-1α is biologically active, only the mature form of IL-1β has any biological activity (Mosley *et al.*, 1987a, 1987b). cDNAs have been isolated encoding a third form of IL-1, IL-1 receptor antagonist (IL-1ra) (Carter *et al.*, 1990; Eisenberg *et al.*, 1990; Hannum *et al.*, 1990). This molecule has homology to both IL-1α and IL-1β and has an affinity for the IL-1 receptors close to that seen for IL-1α and IL-1β, yet elicits no biological response from target cells (Arend *et al.*, 1990; Carter *et al.*, 1990; Eisenberg

et al., 1990; McMahan *et al.*, 1991). Alignments among these three sequences for several species do not indicate which residues are important for activity (Yanofsky and Zurawski, 1990). The three-dimensional structures of IL-1α (Graves *et al.*, 1990) and IL-1β (Gilliland *et al.*, 1987; Priestle *et al.*, 1990) demonstrate the structural similarity of the two molecules but do not suggest which regions of the molecules are responsible for activity.

Deletion and combinatorial mutagenesis have identified residues at the amino terminus of IL-1α which are needed for biological activity (Yanofsky and Zurawski, 1990). However, since the affinities of these mutants for IL-1 receptors were not examined, it is impossible to differentiate whether these mutations affect biological activity, the ability to bind IL-1 receptor, or the structural integrity of the protein. The existence of IL-1ra demonstrates the ability to separate biological activity from binding activity. Although many site-directed mutations of IL-1α and IL-1β have little effect on the function of the proteins (Gronenborn *et al.*, 1988; Kamogashira *et al.*, 1988a, 1988b; Craig *et al.*, 1989), several mutants demonstrate greatly reduced biological activity with little change in affinity for the type I IL-1 receptor (Gehrke *et al.*, 1990; Yamayoshi *et al.*, 1990).

Using a novel method of saturation mutagenesis, random mutations were generated throughout the entire sequence of IL-1α. Assays to determine both biological and binding activity were performed on several thousand mutant proteins. By examining the ratio of biological to binding activity for each mutant and comparing it with the ratio for wild-type IL-1α, regions of IL-1α which affect these two properties differentially were identified.

MATERIALS AND METHODS

Enzymes and Vectors—All restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were obtained from Boehringer Mannheim or New England Biolabs. BBG1, a plasmid bearing a synthetic gene for human IL-1α, was purchased from British Biotechnology. The construction of pPLBBGIL-1α has been described previously (Poindexter *et al.*, 1991). An *Spe*I site was added using site-directed mutagenesis. Plasmid DNA was purified by the alkaline lysis method (Ausubel *et al.*, 1988).

DNA Synthesis—Oligonucleotide cassettes used for the construction of mutants were synthesized on an Applied Biosystems model 380A DNA synthesizer. For saturation mutagenesis each of the four phosphoramidites was contaminated with a small amount of the other three. The phosphoramidites were contaminated at two different levels, 4.2% for the sense strand and 8.4% for the antisense strand (Poindexter *et al.*, 1991). For random mutagenesis, the cassette was synthesized normally except for the substitution of an equimolar mixture of the four phosphoramidites for the three nucleotides making up the chosen codon. Oligonucleotides were purified by polyacrylamide gel electrophoresis on a 40-cm 8% polyacrylamide, 7 M urea gel. Care was taken to excise full-length oligonucleotides, and the

---

§ To whom correspondence should be addressed: Immunex Research and Development Corp., 51 University St., Seattle, WA 98101. Tel.: 206-587-0430; Fax: 206-233-9733.

¶ Present address: Protein Research Laboratories, 10606-8 Camino Ruiz, Suite 281, San Diego, CA 92126.

[1] The abbreviations used are: IL-1, interleukin-1; IL-1ra, interleukin-1 receptor antagonist.

FIG. 1. Coding sequence of human IL-1α gene used in saturation mutagenesis. The DNA sequence downstream from the promoter of pPLBBGIL-1α is shown (accession no. X55445). The coding sequence is represented by *capital letters*. The coding sequence for the IL-1α gene is divided into eight regions, named for the restriction enzymes that border them. The nine restriction enzymes are *Cla*I (C), *Eco*RI (R), *Pst*I (P), *Sst*I (S), *Pvu*II (U), *Bam*HI (B), *Spe*I (S), *Bgl*I (G), and *Hind*III (H). Thus the eight regions are CR, RP, PS, SU, UB, BS, SG, and GH.

```
ClaI (C)
                M  S  A  P  F  S  F  L  S  N  V  K  Y  N  F  M  R
  1 atcgatactATGTCAGCACCTTTTAGCTTCCTGAGCAATGTGAAATACAACTTTATGAGG  60
    EcoRI (R)
    I  I  K  Y  E  F  I  L  N  D  A  L  N  Q  S  I  I  R  A  N
 61 ATCATCAAATACGAATTCATTCTGAACGATGCATTGAACCAGTCTATTATTCGTGCAAAC 120
    PstI (P)
    D  Q  Y  L  T  A  A  A  L  H  N  L  D  E  A  V  K  F  D  M
121 GACCAGTACCTGACTGCAGCAGCCCTGCACAATCTGGACGAAGCAGTTAAATTCGACATG 180
          SstII (S)
    G  A  Y  K  S  S  K  D  D  A  K  I  T  V  I  L  R  I  S  K
181 GGTGCTTACAAGAGCTCGAAAGACGACGCAAAAATCACTGTAATCCTACGTATTTCTAAA 240
    PvuII (U)
    T  Q  L  Y  V  T  A  Q  D  E  D  Q  P  V  L  L  K  E  M  P
241 ACCCAGCTGTATGTAACTGCACAGGATGAAGATCAGCCAGTACTTCTGAAAGAAATGCCT 300
          BamHI (B)
    E  I  P  K  T  I  T  G  S  E  T  N  L  L  F  F  W  E  T  H
301 GAGATCCCGAAGACTATCACTGGATCCGAGACTAACCTGCTGTTCTTCTGGGAAACTCAC 360
          SpeI (S)
    G  T  K  N  Y  F  T  S  V  A  H  P  N  L  F  I  A  T  K  Q
361 GGTACCAAAAACTACTTCACTAGTGTGGCTCATCCGAACCTGTTCATCGCGACAAAACAG 420
          BglI (G)
    D  Y  W  V  C  L  A  G  G  P  P  S  I  T  D  F  Q  I  L  E
421 GACTACTGGGTATGCCTGGCAGGCGGTCCGCCATCGATCACTGACTTCCAGATCCTCGAG 480
          HindIII (H)
    N  Q  A  *  *
481 AACCAAGCATAATAAgatctaagctt 506
```


(A)

(B)
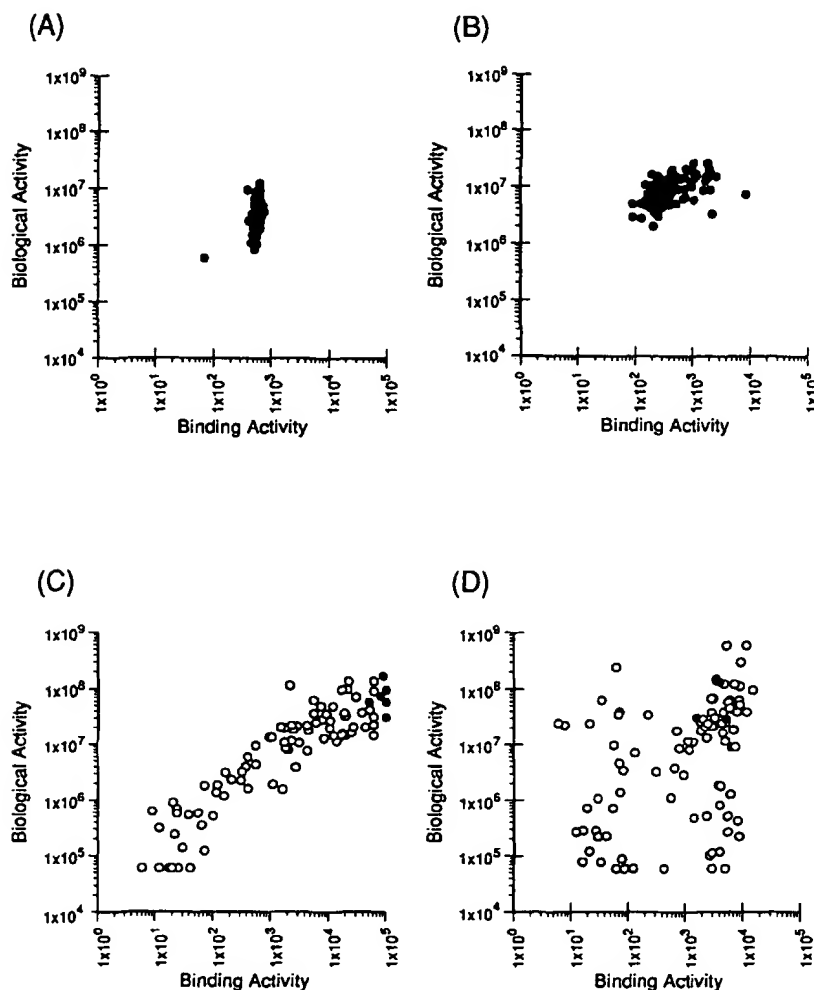
FIG. 2. Ability of biological and binding activity screens to identify mutants with altered activity. *Panel A*, 96 identical samples of wild-type IL-1α. *Panel B*, 96 independent samples of wild-type IL-1α. *Panel C*, 90 mutants of IL-1α from the PS region (*open circles*) and 6 wild-type IL-1α (*closed circles*). *Panel D*, 90 mutants of IL-1α from the RP region (*open circles*) and 6 wild-type IL-1α (*closed circles*). The biological activity is expressed in units/ml, and the binding activity is the reciprocal dilution that results in 50% binding inhibition (see "Materials and Methods").

(C)

(D)

oligonucleotides were deprotected and resuspended in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA).

Assembly and Cloning of Mutagenic Oligonucleotides—The general procedure has been described (Poindexter *et al.*, 1991). Twenty picomoles of each oligonucleotide in a mutagenic cassette were mixed in 20 μl of TE and placed at 65 °C for 15 min. The mixture was allowed to cool slowly to room temperature and then placed on ice. Each

mutagenic cassette had unique ends, allowing them to be ligated into appropriately cleaved vectors. To increase the efficiency of screening, intermediate vectors were constructed for each region. These intermediate vectors contained an irrelevant segment of DNA inserted between the relevant restriction enzyme sites. Insertion of the mutagenic cassette followed by restriction with an enzyme unique to the intermediate plasmid greatly reduced the incidence of vectors without
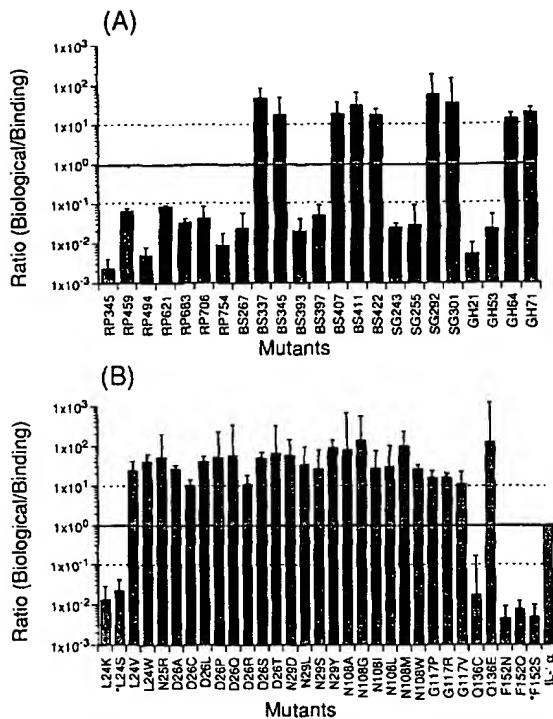
FIG. 3. Mutants with altered activities. The activity ratios were normalized to the internal wild-type IL-1α controls and the mean from at least three screens for mutants with normalized ratios 10-fold greater or less than IL-1α are shown. *Panel A*, proteins generated by saturation mutagenesis. *Panel B*, proteins generated by site-directed random mutagenesis at single amino acid residues. The single letter amino acid code is used to describe the amino acid in wild-type IL-1α, the number of the residue, and the amino acid of the mutant. An *asterisk* indicates single amino acid changes that were also isolated from the saturation mutagenesis screens.

a cassette inserted. The ligation mixture was transformed into GM1[pRK248]. The transformants were screened for insertion of the mutagenic cassette by colony hybridization (Poindexter *et al.*, 1991). Double-stranded sequencing of the vectors with inserts was performed using the dideoxy method (Sanger *et al.*, 1977).

Protein Expression and Analysis—Mutant proteins were produced by using a pH induction protocol (Poindexter and Gayle, 1991). Cells containing recombinant plasmids were inoculated into 24-well plates containing 1 ml of Superbroth (Ausubel *et al.*, 1988), supplemented with M9 minimal salts and 1% glucose. Following overnight growth at 30 °C, the pH of the medium was shifted to 9 by the addition of 5 M NaOH. The cells were grown at 30 °C at pH 9 for 18 h before the cells were pelleted. Cells (40 μl) were spun down in a 96-well plate at 3,000 rpm for 15 min. The cells were resuspended in an equal volume of lysis buffer (125 mM Tris, pH 8, 2% SDS) and then 80 μl of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) was added. The samples were then used for biological or binding assays. Binding activity was determined by the capacity of *Escherichia coli* cell lysates to inhibit the binding of radiolabeled IL-1α to EL4 cells (Mosley *et al.*, 1987b). Biological activity was examined using an EL4 conversion assay (Mosley *et al.*, 1987a). *E. coli* lysates containing wild-type IL-1α were included in every assay as controls. The data were analyzed by a nonlinear least squares fitting routine, and activities were quantified using a standard curve, derived from purified IL-1α, for each biological assay and each binding assay.

Modeling IL-1α Structure—The three-dimensional model of IL-1α used in this study is an all atom protein model built using the Cα coordinates generated from the stereo diagrams of the crystal structure (Graves *et al.*, 1990). The X and Y coordinates for all of the Cα atoms were measured in the user unit using one of the two figures in the stereo diagram. The known standard distance of 3.8 Å, expected between the successive Cα atoms, was used to scale and assign the Z coordinates for all of the Cα atoms. An all atom model of 159 residues

was constructed in FRODO (Jones, 1985) from these 151 Cα coordinates (Leu-7 to Asn-157). The structure was refined in a modified version of Biograf (Peeples and Goldstein, 1989).

Distances between the successive Cα atoms were computed in the X-Y projection by measuring the X and Y coordinates from one of the two stereo diagrams. A value of +1, -1, or 0 was assigned to the Z coordinates of every Cα atom if it was above, below, or in the same depth as the previous Cα atom in the stereo view. The scale factor required to change the user unit to Å unit was computed by equating the computed projection distance between two successive Cα atoms, with a sign of 0, to that of the standard distance of 3.8 Å. The X and Y coordinates of all of the Cα atoms were multiplied with this scale factor, and the projection distance between the successive Cα coordinates was computed. All of the projection distances measured this way will be either less than or equal to 3.8 Å because of the Z flattening. Appropriate Z coordinates were then assigned to each Cα atom such that the computed distance between successive Cα measured 3.8 Å. The computed Z coordinates were multiplied with the sign assigned to them. The cumulative error in the computation of the Z coordinates was corrected by adjusting only the Z coordinates using a wire model. The accuracy of the computed coordinate was tested by superimposing the resulting Cα trace on to the structurally conserved regions of IL-1β structure. The root mean square value for the structurally conserved region is 0.75 Å.

RESULTS

Alteration of the IL-1α Gene by Saturation Mutagenesis—The synthetic IL-1α gene has unique restriction enzyme sites approximately every 60 base pairs, dividing the gene into eight different regions (Fig. 1). The mutants from each region of the molecule are described by the restriction enzymes found at each end of the region. Thus the eight regions are CR (*ClaI-EcoRI*), RP (*EcoRI-PstI*), PS (*PstI-SstI*), SU (*SstI-PvuII*), UB (*PvuII-BamHI*), BS (*BamHI-SpeI*), SG (*SpeI-BglI*), and GH (*BglI-HindIII*). Saturation mutagenesis using cassettes for each region was performed throughout the entire molecule, relying on a technique that results in very low levels of wild-type sequences and roughly equal probabilities of one to five nucleotide changes in any one region (Poindexter *et al.*, 1991). The use of intermediate plasmids in the constructions, along with colony hybridizations (see "Materials and Methods"), greatly improved the yield of recombinant vectors, allowing insert frequencies over 90% to be achieved. More than 3,500 mutants were generated by this approach, encompassing the eight regions of the IL-1α gene (Fig. 1).

In one region (RP), 30 mutants were sequenced to determine accurately the mutation frequencies (Poindexter *et al.*, 1991). Those 30 mutants averaged 2.5 amino acid changes each, and every amino acid that could be altered in this region was found to be changed at least once. Several amino acids had three or four different substitutions. Sequencing mutants in each of the other seven regions did not reveal any deviation from the expected mutation frequency. More than 110 mutants were sequenced, and approximately 70% of the amino acid residues in IL-1α were altered at least once. At this rate of mutagenesis, screening approximately 520 mutants, or roughly 65 mutants from each of the eight regions, should result in a 99% chance that all of the amino acids in IL-1α which could be changed were altered at least once (for a discussion of calculating these probabilities, see Hutchison *et al.*, 1986). Thus, examination of 3,500 mutants should sample multiple mutations at every possible amino acid.

Screening of Mutant IL-1α Proteins—Determining both the biological activity and the ability to inhibit the binding of wild-type IL-1α to the type I IL-1 receptor for each mutant allowed molecules to be identified which affected these two functions differentially. The ratio of biological activity to binding ability was examined. This ratio represents an intrinsic specific activity of the molecule and therefore should be independent of protein concentration. To validate this ap-

FIG. 4. Sequence of mutants that alter activity. The amino acid sequence of the relevant cassettes is shown with the amino acid changes for each mutant underneath. The single letter amino acid code is used.

RP Mutants

RP Mutants	I	L	N	D	A	L	N	Q	S	I	I	R	A	N	D	Q	Y	L	T
RP345			D			M											D		
RP459				E	W														
RP494												M	P	S			N		
RP621					E					T									
RP683								L		S									
RP706					E										M				A
RP754/761		S																	

BS Mutants

BS Mutants	S	E	T	N	L	L	F	F	W	E	T	H	G	T	K	N	Y	F	T
BS267				T									D				F		
BS337					R							Q	V						
BS345				Y							K								P
BS393						P	S									K	F		
BS397							D						D	I					
BS407	A		I				Y										H		
BS411				K							D						D		
BS422								L			V		D						

SG Mutants

SG Mutants	S	V	A	H	P	N	L	F	I	A	T	K	Q	D	Y	W	V	C	L
SG243				Y									L						
SG255									S								L		
SG292											T		H						
SG301				T								N					S		

GH Mutants

GH Mutants	A	G	G	P	P	S	I	T	D	F	Q	I	L	E	N	Q	A
GH21										S							
GH53						L					V	H					
GH64								W	L				K				K
GH71							S	S									

proach, the biological activity and binding activity of 96 identical samples of an *E. coli* lysate containing wild-type IL-1α were determined (Fig. 2A). Plotting the biological activity against the binding activity resulted in a cluster of points, with the greatest error being in the biological activity, presumably because of the greater inherent variation in this assay than in the binding assay. Screening *E. coli* lysates from 96 different inductions, each producing wild-type IL-1α, resulted in the greater scatter with a tendency for the points to cluster along a line whose slope equaled the activity ratio of wild-type IL-1α (Fig. 2B). Altering the concentration of wild-type IL-1α only moves the ratio along this line. Dilutions were performed to verify that the activity seen was linear with respect to IL-1α concentration (data not shown). The assays were able to distinguish levels of biological and binding activity over a range greater than 1,000-fold.

Mutants with a wild-type activity ratio should fall along the same line as wild-type IL-1α controls included in each assay. Proteins with increased biological activity in comparison to the amount of binding activity seen should fall above this line, whereas mutant proteins with decreased amounts of biological activity compared with the binding activity seen should fall below this line. For screening purposes, mutants were determined to have altered activity ratios if there was at least a 10-fold increase or decrease in the ratio relative to wild-type IL-1α for at least three independent sets of assays. In addition, mutants whose *E. coli* lysates lacked activity in either assay were rescreened to verify the lack of activity.

Approximately 1,700 clones were examined from the CR, PS, SU, and UB regions, encompassing more than one-half of the molecule. None of the mutants that demonstrated activity displayed any significant deviation from wild-type levels. These regions have an average of 2.2 amino acid changes per mutant (data not shown), which is not significantly different from the expected mutation rate. Fig. 2C displays a set of typical data for 90 mutants from the PS region of the molecule. The majority of these proteins have activity ratios similar to wild-type IL-1α. The other three regions give similar profiles.

Although most of the 1,800 mutants examined from the other four regions (RP, BS, SG, and GH) had activity ratios within 10-fold of wild-type IL-1α, each region included several mutants that had a ratio that deviated from the wild-type IL-1α ratio by more than 10-fold. An analysis of 90 typical mutants from the RP region is shown in Fig. 2D. There are several mutants in this group which have significantly different activity ratios from wild-type IL-1α. Although several of these mutants failed to maintain an altered activity ratio upon subsequent assays, many continued to have altered activity ratios.

Mutants from saturation mutagenesis with activity ratios 10-fold higher or 10-fold lower than wild-type are shown in Fig. 3A. IL-1α showed itself to be extremely resilient to change. Only 24 unique DNA sequences, out of more than 3,500 examined, produced protein that displayed a significant difference in activity from wild-type (Fig. 3A). This represents less than 0.7% of the mutants examined.

Of the 24 different mutants with altered ratios, 23 had unique amino acid sequences (Fig. 4). RP754 and RP761, although having the same amino acid sequence, have different DNA sequences. This demonstrates the power of this approach since it was possible to identify two independent clones
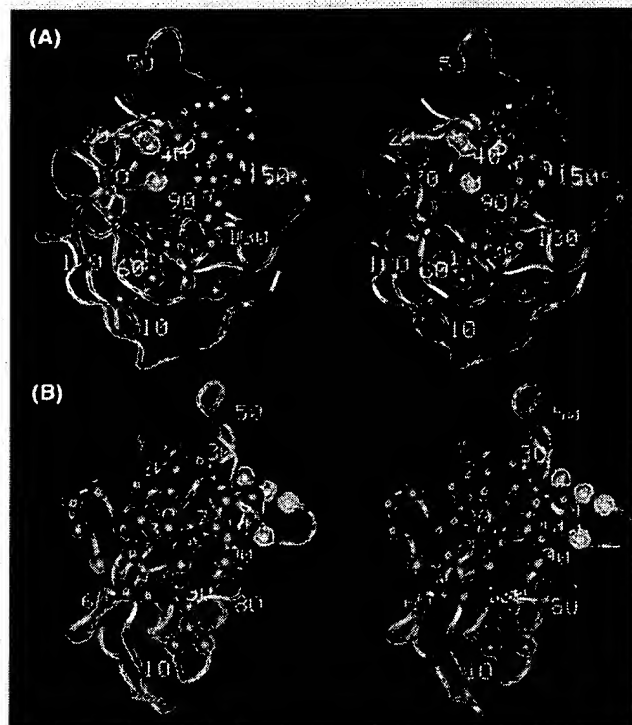
FIG. 5. **Three-dimensional model of IL-1α.** Stereoview showing position of residues that affect the activity of the molecule. *Panel A*, the plane of the figure is roughly perpendicular to the axis of the 12-stranded barrel. *Panel B*, rotated 90° from view in *panel A*. The α-carbon backbone is shown as a *ribbon*, with every 10th residue numbered. The *green spheres* illustrate the major region identified. The *red* and *yellow spheres* indicate the smaller regions made up of residues Glu-106, Asn-108, Leu-109, His-127, and Ile-33, Arg-34, Ala-35, Tyr-39, Thr-41, and respectively.

that had the same phenotype and the same amino acid changes out of a large pool of recombinants. The 23 distinct amino acid sequences contained mutations at a total of 38 amino acid residues. This corresponds to approximately 24% of the molecule.

Random Site-directed Mutagenesis—Because saturation mutagenesis often results in conservative amino acid changes, random mutagenesis was performed at several sites in the molecule. During the synthesis of a particular mutagenic cassette, a random sequence was placed at the codon of interest, allowing all possible amino acids to be produced at this position. Site-directed mutagenesis was performed at residues in the four regions identified by saturation mutagenesis. Leu-24 and Phe-152 were chosen because single amino acid changes introduced at these positions had already been found to alter the activity ratio (*i.e.* mutants RP754 and GH21). Asp-26, Leu-28, Asn-108, Gly-117, and Tyr-121 were examined because more than one mutant had been found which altered these residues. Asn-25 and Gln-136 were studied because they appeared to be conserved hydrophilic residues that were exposed to solvent, according to the crystallographic data (Graves *et al.*, 1990). Asn-29 was also mutated because it was one of the few conserved residues in the RP region of the molecule which was not identified using saturation mutagenesis. Up to 100 mutants at each position were examined. Screening proteins generated by random mutagenesis identified several further single amino acid changes that affected the activity ratios of the molecule (Fig. 3B). Although none of the changes at Leu-28 or Tyr-121 appeared to affect the ratio of biological activity to binding activity, substitutions at

each of the other 8 amino acids produced at least one mutant that altered the activity ratio.

Spatial Location of Mutations—The crystal structures of IL-1α (Graves *et al.*, 1990) and IL-1β (Gilliland *et al.*, 1987; Priestle *et al.*, 1990) have been determined to 2.7 and 2.0 Å, respectively. Using a novel approach, the three-dimensional coordinates were determined from the published structure. The model of IL-1α superimposes on the similar trace from IL-1β with a root mean square difference of 0.75 Å. Residues that were determined by saturation mutagenesis and by random mutagenesis to affect activity ratios are displayed on the model for IL-1α (Fig. 5). Interestingly, these amino acids cluster in three regions. The majority are found along one side of the molecule, encompassing an area of approximately 600 Å².

DISCUSSION

More than 3,500 mutants were generated throughout IL-1α by saturation mutagenesis. The rate of mutagenesis was more than sufficient to produce several amino acid changes at every possible residue in these 3,500 mutants. The biological activity and the ability to inhibit the binding of IL-1α were measured for every mutant. The ratio of biological activity to binding activity gives a measure of the specific activity of each mutant. Antagonists will have low ratios, whereas mutants with high ratios demonstrate enhanced agonist activity.

Most of the molecule could be mutated with little effect on either activity. Combining the data from saturation mutagenesis and site-directed mutagenesis, alterations at only 39 positions resulted in proteins with modified activity ratios. Although mutations at these residues resulted in proteins with activity ratios up to 1,000-fold less than wild-type, it would appear that only a limited number of residues are critically required for activity. Most of the other 139 residues, or 75% of the molecule, may not contribute significantly to the biological activity of the molecule. As much as 68% of IL-1α may have little informational content, allowing a wide variety of amino acids to be substituted with little effect on activity (Zurawski, 1991). This is consistent with the observations reported in this paper.

Fig. 5 shows the spatial locations of the 39 residues changed in mutants with altered activity ratios. Almost all of the amino acid changes were found in β-strands, not in loops. With the exception of amino acids in strands 1 and 12, most of these amino acid residues have not been identified previously as important for activity. More than 75% of the identified amino acid residues are located along one face of the molecule. A substantial number of the mutated residues are located in β-strands 1, 2, 8, 9, 11, and 12. Several residues that may be involved in determining the activity of IL-1α, such as Asp-26, Lys-119, Gln-136, Ile-149, and Asp-151, appear in spatially similar positions in IL-1β.

In addition to this one major region there appear to be two smaller areas: one that includes Glu-106, Asn-108, Leu-109, and His-127, and another involving residues Ile-33, Arg-34, Ala-35, Tyr-39, and Thr-41. The former three amino acids form a small hydrophilic patch at the bottom and slightly behind the large region of mutated residues, whereas the latter amino acids form an exposed patch off to one side, separated from the main region by strands 3 and 4. The identification of three regions important for activity is intriguing. Since the IL-1 receptor is composed of three IgG-like domains, it has been postulated that each of the three domains interacts with a region on IL-1 (Clore *et al.*, 1991). Deletion of any of these three domains greatly reduces the binding of ligand (Dower and Sims, 1990).

```
                                    1                2                3
huIL-1α  S A P F S F L S N V   K Y N F M R I I K Y   E . F I L N D A L N   Q S I I R A . N D Q   Y L T A A A L . H N   47
muIL-1α  S A P Y T Y Q S D L   R Y K L M K L V R Q   K . F V M N D S L N   Q T I Y Q D V D K H   Y L S T T W L . . N
huIL-1β  . . . . . . . . . .   . . . . A P V R S L   N . C T L R D S Q Q   K S L V M S . G P Y   E L K A L H L Q G N   36
muIL-1β  . . . . . . . . . .   . . . . V P I R Q L   H . Y R L R D E Q Q   K S L V L S . D P Y   E L K A L H L N G N
IL-1RA   . . . . . . . . . .   R P S G R K S S K M   Q A F R I W D V N Q   K T F Y L R . N . N   Q L V A G Y L Q G P
                             ●●●      ●●       ●   ●        ●●●●●●                    ● ●       ○○○
                               ○○○    ○          ○       ○

                                    4                5                6                7
huIL-1α  . L D E A V K F D M   G A Y K S S K D D A   K I T V I L R I S K   T Q L Y V T . A Q D   E D Q P V L . L K E   94
muIL-1α  D L Q Q E V K F D M   Y A Y S S G G D D S   K Y P V T L K I S D   S Q L F V S . A Q G   E D Q P V L . L K E
huIL-1β  D M E Q Q V V F S M   S F V Q G E E S N D   K I P V A L G L K E   K N L Y L S C V L K   D D K P T L Q L E S   84
muIL-1β  N I N Q Q V I F S M   S F V Q G E P S N D   K I P V A L G L K G   K N L Y L S C V M K   D G T P T L Q L E S
IL-RA    N V V L E E K I D V   V P I . . E P . . .   . H A L F L G I H G   G K M C L S C V K S   G D E T R L Q L E A
             ○                    ○        ○○○○       ○                  ○○ ○

                                    8                9               10               11
huIL-1α  M P . E I P K T I T   G S E T N L L L F F W   E T H G T K N Y F T   S V A H P N L F I A   T K Q . . D Y W V C   141
muIL-1α  L P . E T P K L I T   G S E T D L I F F W     K S I N S K N Y F T   S A A Y P E L F I A   T K E . . Q S R V H
huIL-1β  V D . P K N Y P K K   K M E K R F V F N K     I E I N N K L E F E   S A Q F P N W Y I S   T S Q A E N M P V F   133
muIL-1β  V D . P K Q Y P K K   K M E K R F V F N K     I E V K S K J V E F E S A E F P N W Y I S   T S Q A E H K P V F
IL-1RA   V N I T D L S E N R   K Q D K R F A F I R     S D S G P T T S F E   S A A C P G W F L C   T A M E A D Q P V S
             ○○      ○        ●  ●●      ●●          ●  ●●●●  ●●  ●          ●          ●           ●●     ●    ●●
                                             ○             ○   ○

                                    12
huIL-1α  L A G . . . G P P S   I T D F Q I L E N Q   A   159
muIL-1α  L A R . . . G L P S   M T D F Q I S . . .   .
huIL-1β  L G G T K G G Q D     I T D F T M Q F V S   S   153
muIL-1β  L G N N S . . G Q D   I I D F T M E S V S   S
IL-1RA   L T N M P D E G V M   V T K F Y F Q E D E   .
                ●●●     ●    ●●●
                            ○   ○   ○
```

FIG. 6. **Alignment of human and murine IL-1α, human and murine IL-1β, and IL-1 receptor antagonist.** Residues that are conserved in at least three of the sequences are *boxed*. The β-strands for IL-1α are shown above the sequence. The numbering for human IL-1α and IL-1β is displayed on the *right*. *Closed circles* indicate amino acid residues that were changed in IL-1α mutants with altered phenotypes. *Open circles* indicate residues in human IL-1β which have been postulated to interact with the type I IL-1 receptor.

Using site-directed mutagenesis, single amino acid changes that decrease or increase the ratio of biological activity to binding ability were found at several positions in the molecule. At most of these locations only the substitution of specific amino acids had any impact on activity. Screening up to 100 isolates revealed only a small number of specific amino acid substitutions at Leu-24, Asn-25, Asn-29, Gly-117, Gln-136, and Phe-152 which altered the activity ratio. Substitutions at Asp-26 and Asn-108 were exceptions. The presence of any of 10 amino acids at Asp-26 altered the activity ratio. Changes at Asp-26 which reduce the amount of biological activity have been reported (Yamayoshi *et al.*, 1990). This may indicate that an Asp residue at position 26 is required for wild-type activity. Several hydrophilic substitutions at Asn-108 did not appear to affect biological activity (Zurawski, 1991). However, placing hydrophobic residues at this position did affect the ratio, with a relative increase in the biological activity.

Few other reports of IL-1α mutagenesis have distinguished between changes that affect biological activity and those that also alter the interactions of the protein with the receptor. Mutations at Asp-151 differentially affect biological activity and binding ability (Yamayoshi *et al.*, 1990). Examination of a large group of IL-1α mutants has previously revealed the importance of Asp-26 and Asp-151, but only the effects on biological activity were studied (Kawashima *et al.*, 1992). Random combinatorial mutagenesis has identified amino acids in which substitutions have no effect on biological activity (Yanofsky and Zurawski, 1990). The lack of muta-

tions at certain residues was used to infer the importance of these residues for biological activity. There was, however, no demonstration of the ability of these mutants to interact with the IL-1 receptor. Since only biological activity was examined, it is difficult to determine whether the constraints on amino acid residues were functional in nature or whether there were structural constraints as well.

Sequence alignments of human and mouse IL-1α and IL-1β are shown in Fig. 6. These molecules bind to the type I IL-1 receptor and are active in the EL4 conversion assay. Inclusion of IL-1ra, a molecule that also binds to IL-1 receptor but produces no biological activity, in the alignment does not immediately reveal any obvious region that is responsible for the uncoupling of biological activity and the ability to bind receptor. The 39 amino acids identified in this report are also shown. Although many of these amino acids are found toward the COOH terminus of the molecule (which is the most highly conserved region of the molecule), there does not appear to be much selection for conserved residues. In fact, regions in which few amino acids are conserved, such as in strand 2 or strand 8, have several residues that appear to be important for activity. Although there are no data demonstrating that IL-1α and IL-1β interact with the type I IL-1 receptor in the same fashion, residues of IL-1β which have been shown to be important for biological activity are found in positions homologous to some of the residues identified for IL-1α (Gehrke *et al.*, 1990; Ju *et al.*, 1991).

Interestingly, residues of IL-1β which have been shown to

influence binding to the type I IL-1 receptor or have been proposed to interact with the receptor (Clore *et al.*, 1991; Grenfell *et al.*, 1991; Labriola-Tompkins *et al.*, 1991; Veerapandian *et al.*, 1992) are generally located outside the regions of IL-1α shown in Fig. 5. Out of 45 amino acids postulated to be involved in binding of IL-1β only 9 overlap with residues identified in this study (Fig. 6). IL-1α and IL-1β either have different regions interacting with the receptor, or there is a large region in IL-1α and IL-1β which is required for biological activity but not for high affinity interactions with the receptor.

A superfamily of molecules with protein folding similar to IL-1 has been proposed (Graves *et al.*, 1990; Murzin *et al.*, 1992). This superfamily includes certain proteinase inhibitors and heparin-binding growth factors, such as fibroblast growth factor. Two forms of fibroblast growth factor have been shown to fold in a very similar fashion to IL-1α and IL-1β (Ago *et al.*, 1991; Eriksson *et al.*, 1991; Zhang *et al.*, 1991; Zhu *et al.*, 1991), displaying 12 β-strands with a pseudo 3-fold symmetry. Several regions of the fibroblast growth factor molecule have been identified which are important for activity. These areas occupy spatially similar regions of the fibroblast growth factor three-dimensional structure as the amino acids of IL-1α identified by saturation mutagenesis. The modes of interaction between members of the IL-1 superfamily and their respective receptors may involve similar regions of the folded protein.

The manner in which IL-1 generates a biological response is complicated. There are two different ligands, an IL-1 antagonist and two forms of IL-1 receptor (Dower *et al.*, 1990). There is evidence for multiple pathways of signal transduction (for review see Sims *et al.*, 1993). The effect of IL-1 on a particular cell type may depend on the receptor found on the cell and which signaling pathway is being used. Analysis of the effect of these mutations on the different biological responses may be helpful in further elucidation of IL-1 signal transduction.

REFERENCES

Ago, H., Kitagawa, Y., Fujishima, A., Matsuura, Y., and Katsube, Y. (1991) *J. Biochem.* **110**, 360–363

Arend, W. P., Welgus, H. G., Thompson, R. C., and Eisenberg, S. P. (1990) *J. Clin. Invest.* **85**, 1694–1697

Auron, P. E., Warner, S. J., Webb, A. C., Cannon, J. G., Bernheim, H. A., McAdam, K. J., Rosenwasser, L. J., LoPreste, G., Mucci, S. F., and Dinarello, C. A. (1987) *J. Immunol.* **138**, 1447–1456

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. J., Smith, J. A., and Struhl, K. (1988) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York

Carter, D. B., Deibel, M. J., Dunn, C. J., Tomich, C. S., Laborde, A. L., Slightom, J. L., Berger, A. E., Bienkowski, M. J., Sun, F. F., McEwan, R. N., Harris, P. K. W., Yem, A. Y., Waszak, G. A., Chosay, J. G., Sieu, L. C., Hardee, M. M., Zurcher-Neely, H. A., Reardon, I. M., Heinrikson, R. L., Truesdell, S. E., Shelly, J. A., Eessalu, T. E., Taylor, B. M., and Tracey, D. E. (1990) *Nature* **344**, 633–638

Clore, G. M., Wingfield, P. T., and Gronenborn, A. M. (1991) *Biochemistry* **30**, 2315–2323

Craig, S., Pain, R. H., Schmeissner, U., Virden, R., and Wingfield, P. T. (1989) *Int. J. Pept. Protein Res.* **33**, 256–262

Dower, S. K., and Sims, J. E. (1990) in *Cellular and Molecular Mechanisms of Inflammation* (Cochrane, C. G., and Gimbrone, J. M. A., eds) pp. 137–172, Academic Press, New York

Dower, S. K., Kronheim, S. R., Hopp, T. P., Cantrell, M., Deeley, M., Gillis, S., Henney, C. S., and Urdal, D. L. (1986) *Nature* **324**, 266–268

Dower, S. K., Bomzstyk, K., and Sims, J. E. (1990) *Prog. Clin. Biol. Res.* **349**, 241–249

Durum, S. K., Schmidt, J. A., and Oppenheim, J. J. (1985) *Annu. Rev. Immunol.* **3**, 263–287

Eisenberg, S. P., Evans, R. J., Arend, W. P., Verderber, E., Brewer, M. T., Hannum, C. H., and Thompson, R. C. (1990) *Nature* **343**, 341–346

Eriksson, A. E., Cousens, L. S., Weaver, L. H., and Matthews, B. W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3441–3445

Gehrke, L., Jobling, S. A., Paik, L. S., McDonald, B., Rosenwasser, L. J., and Auron, P. E. (1990) *J. Biol. Chem.* **265**, 5922–5925

Gilliland, G. L., Winborne, E. L., Masui, Y., and Hirai, Y. (1987) *J. Biol. Chem.* **262**, 12323–12324

Graves, B. J., Hatada, M. H., Hendrickson, W. A., Miller, J. K., Madison, V. S., and Satow, Y. (1990) *Biochemistry* **29**, 2679–2684

Grenfell, S., Smithers, N., Witham, S., Shaw, A., Graber, P., and Solari, R. (1991) *Biochem. J.* **280**, 111–116

Gronenborn, A. M., Wingfield, P. T., McDonald, H. R., Schmeissner, U., and Clore, G. M. (1988) *FEBS Lett.* **231**, 135–138

Hannum, C. H., Wilcox, C. J., Arend, W. P., Joslin, F. G., Dripps, D. J., Heimdal, P. L., Armes, L. G., Sommer, A., Eisenberg, S. P., and Thompson, R. C. (1990) *Nature* **343**, 336–340

Hutchison, C. A. I., Nordeen, S. K., Vogt, K., and Edgell, M. H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 710–714

Jones, T. A. (1985) *Methods Enzymol.* **115**, 157–171

Ju, G., Labriola-Tompkins, E., Campen, C. A., Benjamin, W. R., Karas, J., Plocinski, J., Biondi, D., Kaffka, K. L., Kilian, P. L., Eisenberg, S. P., and Evans, R. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 2658–2662

Kamogashira, T., Masui, Y., Ohmoto, Y., Hirato, T., Nagamura, K., Mizuno, K., Hong, Y. M., Kikumoto, Y., Nakai, S., and Hirai, Y. (1988a) *Biochem. Biophys. Res. Commun.* **150**, 1106–1114

Kamogashira, T., Sakaguchi, M., Ohmoto, Y., Mizuno, K., Shimizu, R., Nagamura, K., Nakai, S., Masui, Y., and Hirai, Y. (1988b) *J. Biochem. (Tokyo)* **104**, 837–840

Kawashima, H., Yamagishi, J.-i., Yamayoshi, M., Ohue, M., Fukui, T., Kotani, H., and Yamada, M. (1992) *Protein Eng.* **5**, 171–176

Labriola-Tompkins, E., Chandran, C., Kaffka, K. L., Biondi, D., Graves, B. J., Hatada, M., Madison, V. S., Karas, J., Kilian, P. L., and Ju, G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 11182–11186

March, C. J., Mosley, B., Larsen, A., Cerretti, D. P., Braedt, G., Price, V., Gillis, S., Henney, C. S., Kronheim, S. R., Grabstein, K., Conlon, P. J., Hopp, T. P., and Cosman, D. (1985) *Nature* **315**, 641–647

McMahan, C. J., Slack, J. L., Mosley, B., Cosman, D., Lupton, S. D., Brunton, L. L., Grubin, C. E., Wignall, J. M., Jenkins, N. A., Brannan, C. I., Copeland, N. G., Huebner, K., Croce, C. M., Cannizzarro, L. A., Benjamin, D., Dower, S. K., Spriggs, M. K., and Sims, J. E. (1991) *EMBO J.* **10**, 2821–2832

Mosley, B., Dower, S. K., Gillis, S., and Cosman, D. (1987a) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 4572–4576

Mosley, B., Urdal, D. L., Prickett, K. S., Larsen, A., Cosman, D., Conlon, P. J., Gillis, S., and Dower, S. K. (1987b) *J. Biol. Chem.* **262**, 2941–2944

Murzin, A. G., Lesk, A. M., and Chothia, C. (1992) *J. Mol. Biol.* **223**, 531–543

Peeples, W. J., and Goldstein, P. (1989) *Cytobios* **58**, 109–123

Poindexter, K., and Gayle, R. B., III. (1991) *Gene (Amst.)* **9**, 125–130

Poindexter, K., Jerzy, R., and Gayle, R. B., III. (1991) *Nucleic Acids Res.* **19**, 1899–1904

Priestle, J. P., Schar, H. P., and Grutter, M. G. (1990) *Prog. Clin. Biol. Res.* **349**, 297–307

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467

Sims, J. E., Bird, T. A., Giri, J. G., and Dower, S. K. (1993) *IL-1 Signal Transduction: Signal Transduction through Growth Factor Receptors* (Kitagawa; Y., ed), Jai Press, Greenwich, in press

Veerapandian, B., Gilliland, G. L., Raag, R., Svensson, A. L., Masui, Y., Hirai, Y., and Poulos, T. L. (1992) *Proteins Struct. Funct. Genet.* **12**, 10–23

Yamayoshi, M., Ohue, M., Kawashima, H., Kotani, H., Ilda, M., Kawata, S., and Yamada, M. (1990) *Lymphokine Res.* **9**, 405–413

Yanofsky, S. D., and Zurawski, G. (1990) *J. Biol. Chem.* **265**, 13000–13006

Zhang, J. D., Cousens, L. S., Barr, P. J., and Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3446–3450

Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T., and Rees, D. C. (1991) *Science* **251**, 90–93

Zurawski, G. (1991) *Trends Biotechnol.* **9**, 250–257